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RESEARCH PAPER

Biomaterial-Based Vaccine Induces Regression of Established Intracranial Glioma in Rats

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ABSTRACT

Purpose The prognosis for glioma patients is poor, and development of new treatments is critical. Previously, we engineered polymer-based vaccines that control GM-CSF, CpG-oligonucleotide, and tumor-lysate presentation to regulate immune cell trafficking and activation, which promoted potent immune responses against peripheral tumors. Here, we extend the use of this system to glioma.

Methods Rats were challenged with an intracranial injection of glioma cells followed (1 week) by administration of the polymeric vaccine (containing GM-CSF, CpG, and tumor-lysate) in the tumor bed. Control rats were treated with blank matrices, matrices with GM-CSF and CpG, or intra-tumoral bolus injections of GM-CSF, CpG, and tumor lysate. Rats were monitored for survival and tested for neurological function.

Results Survival studies confirmed a benefit of the polymeric vaccine as 90% of vaccinated rats survived for >100 days. Control rats exhibited minimal benefit. Motor tests revealed that vaccination protected against the loss of forelimb use produced by glioma growth. Histological analysis quantitatively confirmed a robust and rapid reduction in tumor size. Long-term immunity was confirmed when 67% of survivors also survived a second glioma challenge.

Conclusions These studies extend previous reports regarding this approach to tumor therapy and justify further development for glioma treatment.

KEY WORDS brain tumor · cancer vaccine · glioma · immunotherapy · PLG

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INTRODUCTION

Brain tumors, particularly gliomas, are a devastating form of cancer with few treatment options. The median survival after surgery and radiation therapy alone is nine months, with approximately 10% of patients surviving for two years (1). Systemic chemotherapy is minimally effective (1–3). The ability of the immune system to react specifically against tumors in the CNS (2,3) and to obtain long-lasting memory that may, ultimately, prevent tumor recurrence (4) has inspired the development of immunotherapies that seek to reprogram anti-glioma responses (2–4). Vaccine approaches to cancer immunotherapy involve the introduction of tumor-associated antigens at the vaccine site, where they are processed by the cell-mediators of immunity, dendritic cells (DCs), that translate these antigenic signals into the priming of cytotoxic T-cells to kill tumor cells (5). This desired effect is highly dependant on the presence of immunostimulatory signals within the microenvironment of the antigen-DC encounter. Danger signals common to pathogenic infection, such as lipopolysacharides or cytosine-guanosine (CpG) sequences in bacterial DNA, are critical in promoting DC activation and are often co-delivered with antigen to amplify the induction of specific immune responses by vaccines (6).

Investigative methods in cancer vaccination, utilize products formulated for bolus delivery (for example, antigen, adjuvants, or activated DCs) and can enhance

antigen targeting to DCs and/or expansion of antigen-specific CTLs, but they lack control over DC fate *in vivo* and induce transient immunostimulation, secondary to biodegradation and rapid systemic clearance that limits bioavailability (5,7–9). Consequently, clinical trials with current brain cancer vaccines have been incapable of producing persistent CTL attacks that reproducibly cause tumor regression and a survival benefit (10,11). Biomaterials of many different types are now routinely modified to control the bioavailability, kinetics and localization of therapeutic molecules (12) and have been utilized in the brain (13), introducing the possibility of exploiting these materials for brain cancer immunotherapy. We recently described the development of three-dimensional, macroporous polymer matrices that regulate the trafficking and activation of the DCs (14). These implantable matrices spatially and temporally control the *in vivo* presentation of cytokines, tumor antigens and danger signals (14,15). Fabricated with GM-CSF, a CpG oligonucleotide (ODN), and tumor lysate, these polylactide-co-glycolide (PLG) matrices release GM-CSF into the surrounding tissue to recruit host DCs (14) following subcutaneous implantation. CpG-rich ODNs, which act as danger signals, and antigen (tumor lysate incorporated within the PLG) are embedded in the matrix or slowly released to matrix resident DCs (Fig. 1) to program DC development and maturation. The coordination of DC activation induced by these biomaterial-based vaccines promotes potent, prolonged, and specific cytotoxic, T-cell-mediated immunity that has been shown to eradicate large peripheral tumors in mice (14,15). Here, we demonstrate, for the first time, that a one-time application of this biomaterial vaccine can completely eradicate established gliomas in rats and that the matrix system was required to confer a therapeutic benefit at the dose levels utilized in this study.

MATERIALS AND METHODS

Cell Culture and Animals

Seventy-nine adult Sprague-Dawley rats (Harlen, Inc.) 2–3 months old, weighing 250–300 g were used. C6 glioma cells from rat (ATCC, CCL-107) were cultured in F-12K

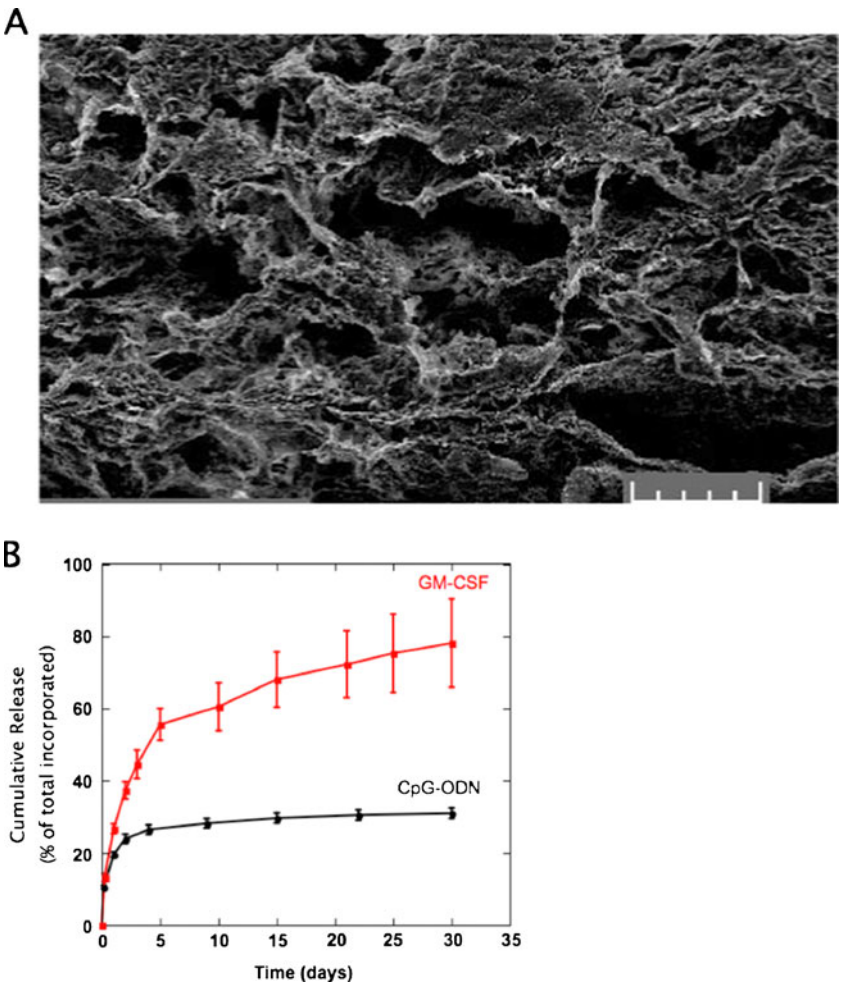

Fig. 1 (**A**) SEM micrograph of a cross-section of a macroporous, PLG vaccine. Scale bar = 200 μm. (**B**) The cumulative release of GM-CSF ($n = 8$) and CpG-ODN ($n = 5$) from PLG vaccines over time with incubation at 37° in PBS *in vitro*. Values represent mean (+/−SD).

Medium (ATCC, 30-2004), 2.5% fetal bovine serum and 15% horse serum at 37°C with5% CO_2.

Matrix Fabrication

A 85:15, 120 kD copolymer of D,L-lactide and glycolide (PLG) (Alkermes, Cambridge, MA) was utilized in a previously described (14) gas-foaming process to form porous PLG matrices (Fig. 1). PLG microspheres encapsulating GM-CSF were first made using standard double emulsion (16). PLG microspheres were then mixed with 150 mg of the porogen, sucrose (sieved to a particle size between 250 μm and 425 μm), and compression molded into 8.5 mm diameter and 1 mm-thick discs (15). The resulting disc was allowed to equilibrate within a high-pressure CO_2 environment, and a rapid reduction in pressure caused the polymer particles to expand and fuse into an interconnected structure. The sucrose was leached from the scaffolds by immersion in water, yielding scaffolds that were 90% porous (Fig. 1). To incorporate tumor lysates into PLG scaffolds, biopsies of C6 gliomas tumors that had grown for 17 days in the brains of rats ($N=9$) were digested in collagenase (250 U/ml) (Worthington, Lakewood, NJ) and suspended at a concentration equivalent to 10^7 cells per ml after filtration through 40 μm cell strainers. The tumor cell suspension was subjected to four cycles of rapid freeze in liquid nitrogen, thawed (37°C) and then centrifuged at 400 rpm for 10 min. The supernatant (1 ml) containing tumor lysates was collected, incubated with the PLG microspheres for 10 min at room temperature, frozen, and then lyophilized, and the resulting mixture was used to make PLG scaffold-based cancer vaccines. To incorporate CpG-ODNs into PLG scaffolds, CpG-ODN 1826, 5'-tcc atg acg ttc ctg acg tt-3', (Invivogen, San Diego, CA) was first condensed with poly(ethylenimine) (PEI, *M*n ~60,000, Sigma Aldrich) molecules by dropping ODN-1826 solutions into PEI solution, while vortexing the mixture (in PBS; pH = 7.2) (15). The charge ratio between PEI and CpG-ODN (NH3+:PO4–) was kept constant at seven during condensation. PEI-CpG-ODN condensate solutions were then vortexed with 60 μl of 50% (wt/vol) sucrose solution, lyophilized and mixed with dry sucrose to a final weight of 150 mg. The dry sucrose containing PEI-CpG-ODN condensate was then mixed with blank or GM-CSF/tumor lysate-loaded PLG microspheres to make PLG cancer vaccines. For intracranial transplantation, the PLG-scaffold discs were cut into 1 mm by 2 mm by 3 mm PLG vaccines.

Incorporation and Release Kinetics

To assess GM-CSF incorporation and bioactivity in PLG vaccines, the device was placed in 98% Glacial Acetic Acid/0.1% Bovine Serum Albumin and vortexed to solubilize the PLGA polymer. Methylene Chloride was added, and the mixture was vortexed. Phosphate-Buffered Saline/0.1% Bovine Serum Albumin was added and the mixture vortexed. The mixture was centrifuged to separate the aqueous/organic layers. The aqueous layer containing the GM-CSF was removed and analyzed for GM-CSF concentration using ELISA (R&D systems).

To assess CpG-ODN incorporation in the device, the vaccine was placed in a high salt extraction buffer to release the oligonucleotide from the matrix. The buffer was applied to a silica mini-prep column to separate the oligonucleotide from other components of the device. The post-column sample was dialyzed in Tris-EDTA buffer and then analyzed for oligonucleotide content and bioactivity using the nucleic acid stain, Oligreen (Sigma).

To measure the total protein incorporated (tumor lysate) into the PLG vaccines, 1.0 N NaOH was utilized to dissolve the PLGA scaffold and solubilize the protein incorporated into the device. The total device protein was quantified using the Micro BCA Protein Assay Kit.

To determine the kinetics of GM-CSF and CpG-ODN release from PLG scaffolds, ^{125}I-labeled hr-GM-CSF (Perkin Elmer) and P^{33}-CpG-ODN 1826 (oligofactory; radiolabeled at Perkin Elmer) were utilized as a tracer, and these PLG vaccines with radiolabelled components were placed in 3 ml of Phosphate Buffer Solution (PBS) in an incubator (37°C) (14,17). At various time-points, the PBS release media was collected and replaced with fresh media. The amount of ^{125}I-hr-GM-CSF and P^{33}-CpG-ODN released from the scaffolds was determined at each time point by counting the radioactivity of the removed media in a 1470 wizard gamma counter (Perkin Elmer) or a tri-carb 2810TR scintillation counter (Perkin Elmer), respectively, and normalizing the result to the total ^{125}I-GM-CSF or P^{33}-CpG-ODN incorporated into the scaffolds.

Surgery and Study Design

Rats were anesthetized with isoflurane (3–4%) and positioned in a stereotaxic instrument (Kopf, Tujunga CA), and cells were injected into the striatum at the following coordinates: 0.5 mm anterior to Bregma, 2.5 mm lateral to the sagittal suture, and 5.5 mm below the cortical surface. A total of 2×10^5 cells were injected in 5 ul of PBS. One week after tumor implantation, the animals were anesthetized, and a 1 mm wide, 2 mm thick, and 3 mm long scaffold was implanted into the tumor bed. Animals were randomly divided into the following groups: (1) glioma only ($N=10$), (2) glioma + blank matrices ($N=10$), (3) glioma + PLG vaccine [GM-CSF, CpG, and tumor lysate] ($N=10$), (4) glioma + GM-CSF/CpG containing matrices

(N=10), (5) glioma + bolus GM-CSF, CpG, and tumor lysate. Bolus injections were performed using procedures identical to the C6 injections. Animals were monitored daily, and any animal exhibiting distress or morbidity was euthanized to determine lifespan. Animals surviving 100 days following tumor implantation received a second re-challenge by injecting glioma cells into the contralateral hemisphere. A set of rats (N=10) received glioma cells only. All animals surviving the tumor re-challenge were sacrificed 110 days later for histological evaluation.

Neurological Evaluation

To assess the functional consequences of tumor growth and any benefits of the vaccine, we tested the animals in the lifespan studies using two neurological tests, sensitive to striatal damage (18). Animals were tested for neurological (motor) function 20 days after tumor inoculation. The first test, the cylinder test, tested the ability of animals to make left and right forepaw contacts with the wall of a 20 cm diameter acrylic cylinder (20 contacts total). The second test was the forelimb placing test, which assesses the rat's ability to place a forepaw on a table when placed parallel to the edge of the table-top using ten trials per forepaw.

Histology

Histology was used to confirm that the PLG vaccine induced the regression of C6 gliomas. All of the long-term survivors from the lifespan studies were sacrificed, and the previous implant sites were examined in paraffin-embedded H&E-stained sections, as described previously (19). Briefly, separate sets of animals were used to determine the effects of the PLG vaccine on tumor growth. Rats received an intracerebral injection of glioma cells followed seven days later by implantation of either a blank polymer matrix (N=5) or a PLG vaccine (N=5) as described above. Seventeen days after tumor inoculation these animals were sacrificed, the brains were stained with H&E, and tumor sizes were quantified.

RESULTS

Matrix Fabrication and Release Kinetics

The cross-section of the macroporous PLG vaccine is shown in an SEM micrograph in Fig. 1A. As previously reported, the matrices were largely porous, isoreticulated scaffolds with an overall porosity of approximately 75%. Glioma vaccines contained 26±1.0 μg of protein derived from glioma tumor lysate, 440±4.0 ng of GM-CSF, and 7.3±0.3 μg of CpG-ODN. The release of radiolabelled GM-CSF and CpG-ODN was similar during the first 24 h, with approximately 20% of CpG-ODN and 25% of GM-CSF released. Total GM-CSF release was more rapid and sustained, with approximately 60% released during the first 10 days and continuing until 80% was released within 30 days. In contrast, the majority of CpG-ODN was retained within the matrix, with cumulative release peaking at approximately 30% within 15 days and stabilizing thereafter (Fig. 1B).

Effects of PLG Vaccines on Survival of C6 Glioma-Bearing Rats

The glioma was uniformly fatal to all non-treated animals (Fig. 2B, Table I). Implantation of blank matrices did not impact survival, but PLG vaccines dramatically increased median and maximum survival (>955% and >840%, respectively) relative to controls, with 90% of the animals surviving for >100 days. Implantation of matrices containing GM-CSF and CpG oligonucleotide alone or a bolus injection of GM-CSF, CpG and tumor cell lysate modestly impacted survival (Fig. 2B; Table I), but no long-term survivors were noted. A persistent immunological memory was established in the surviving rats, as 67% of them survived a second re-challenge of C6 gliomas for another 110 days (Fig. 2C). Those animals that did succumb to the re-challenge survived significantly longer (~ 50% longer) than controls.

Effects of PLG Vaccines on Tumor Growth

The enhanced survival produced by the vaccines was mirrored by significant histological regression of tumors (Fig. 3). When analyzed 17 days post-tumor-injection, H&E histology revealed that, in controls, the tumors had grown in all animals and in some cases grew to encompass much of the injected striatum (Fig. 3A). The tumors were notably smaller in animals treated with PLG vaccines, with no discernable tumor mass in 40% (2/5) of the animals (Fig. 3B). Quantitative determinations of tumor area (Fig. 3C) at this time-point revealed a >50% decrease in tumor area (3.97 mm^2 *vs* 1.58 mm^2).

Neurological Evaluation

The potency of the PLG vaccine was confirmed by the performance of neurological tests. (Fig. 4). In both cylinder and placement tests of limb function, significant impairments in performance were noted in controls that were obviated by implantation of PLG vaccines. Indeed, the vaccines completely prevented the impairments in forepaw use resulting in performance that was indistinguishable from the intact, ipsilateral forepaw. Slight benefits were

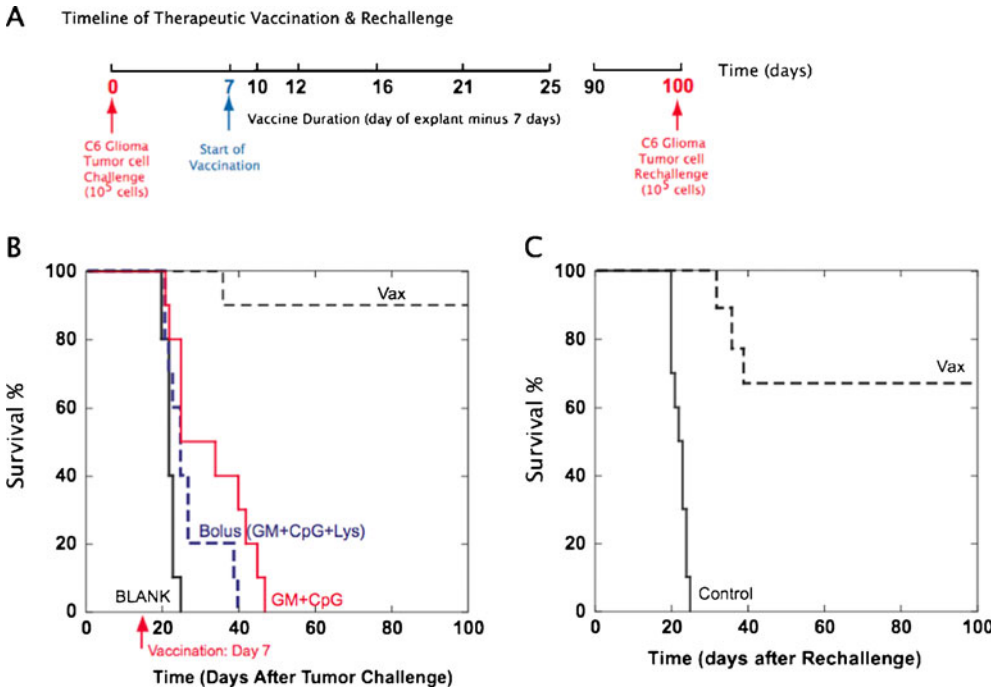

Fig. 2 Therapeutic vaccination of rats bearing glioma tumors and protection against rechallenge. (**A**) Schematic of PLG vaccine regimen for therapeutic C6 glioma models in Sprague-Dawley rats and subsequent re-challenge. (**B**) A comparison of the survival time of tumor-bearing rats treated with blank PLG matrices (BLANK), vaccines incorporating GM-CSF and CpG (GM + CpG), PLG vaccines containing GM-CSF, CpG and glioma lysates (Vax), or a bolus injection of GM-CSF, CpG, and glioma lysates. (**C**) The survival time of rats re-challenged with C6 glioma cells at day 100 after the initial innoculation with C6 glioma cells.

obtained with matrices containing GM-CSF + CpG oligonucleotide, and no benefits were observed in animals receiving a bolus injection.

DISCUSSION

The poor prognosis for glioma patients has resulted in a continued effort to develop novel and innovative treatments that center on the ability to target all intracranial neoplastic foci left behind after surgical resection of the primary tumor while minimizing collateral damage to the surrounding, normal brain. This may be achieved by developing immunotherapeutic approaches that enable antigen-specific, tumor cell attack while the induction of immune memory offers prevention of recurrent brain tumors. To date, research into the use of biomaterials for brain cancer immunotherapy has been limited and focused on the controlled delivery of adjuvants that may up-regulate local immune responses. Immunostimulatory cytokines, such as interleukins (IL-2 and IL-12), and GM-CSF (7) have been encapsulated into biomaterial particles to sustain their release or to co-deliver adjuvants with chemotherapeutics (13,20–22) which can promote immune cell infiltration into the sites of resected or dying tumors, slow the onset of tumor recurrence, and enhance survival in animal models. Biomaterial-based vaccine systems, mostly in particulate form, have been shown to enhance the targeting and duration of antigen delivery and immune cell activation (23), which amplified immunity to peripheral tumors in animals, but they have yet to be applied in the CNS.

This report demonstrates that the coordinated, intracranial presentation of GM-CSF, CpG-ODN danger signals, and glioma lysates from three-dimensional PLG matrices can cause the regression of gliomas and confer 90% long-term survival rates (100 days) in rats that would otherwise die (~23 days). Immune memory was established as a second glioma-cell challenge revealed attenuated tumor progression resulting in 67% long-term survival. Moreover, neurological tests demonstrated that glioma-bearing rats treated with PLG vaccines maintained limb function and did not exhibit any morbidity associated with the intracra-

Table I Survival Following Implantation of PLG Vaccines into C6 Glioma-Bearing Rats

Treatment	Median survival (days)	% Control (tumor only)	Maximum survival (days)	% change (tumor only)
Glioma only	22	NA	25	NA
Blank matrix	22	100%	25	0%
PLG Vaccine (GM-CSF + CpG + tumor lysate)	>210	>955%	>210	>840%
No lysate matrix (GM-CSF + CpG only)	24	109%	46	184%
Bolus injection (GM-CSF + CpG + tumor lysate)	25	114%	40	160%

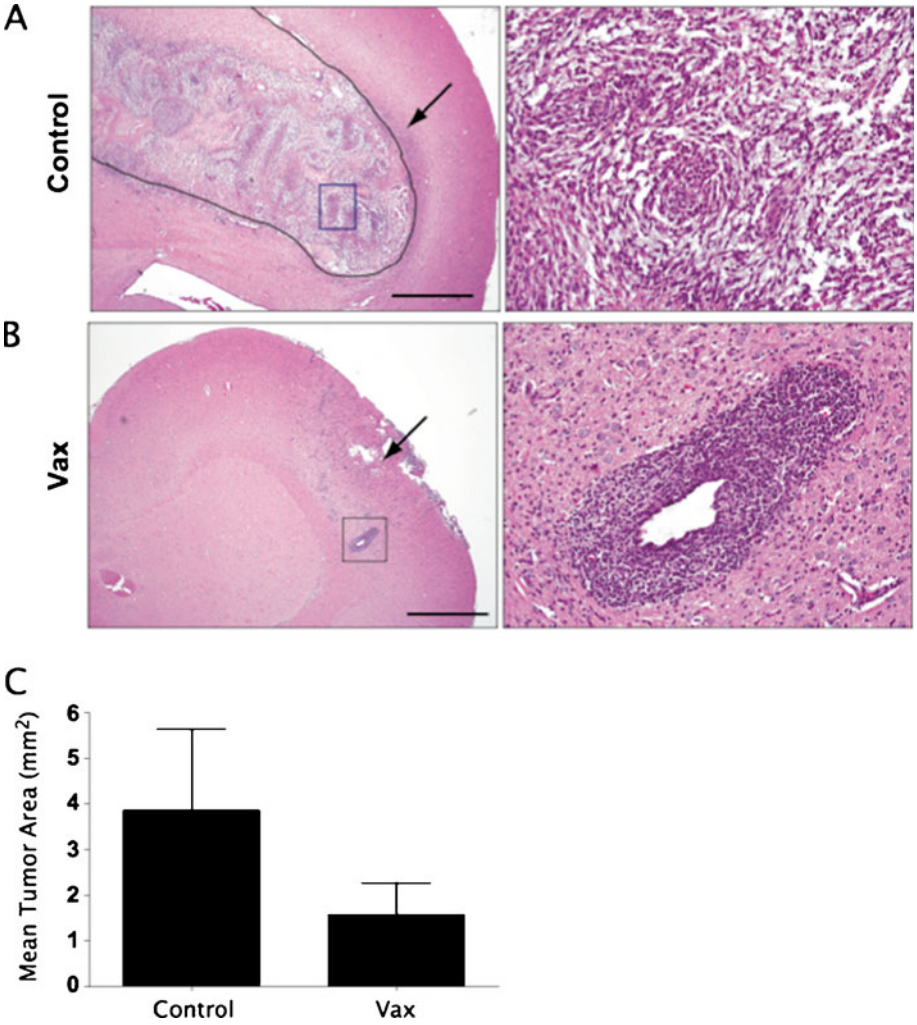

Fig. 3 Reduction in tumor size following implantation of PLG vaccines (**A**) Low (10×) and high (200×) power H&E stained sections of brains 17 days after C6 glioma cell challenge in untreated control animals and (**B**) animals treated with PLG vaccines. The arrow in (**B**) points to the location of matrix implantation and contains areas of gliosis and inflammatory cell infiltrates. A small, residual pocket of glioma cells is outlined in a vaccinated rat. (**C**) The mean (+/−SD) tumor areas in control and vaccinated rats (Vax). Scale bar—1 mm.

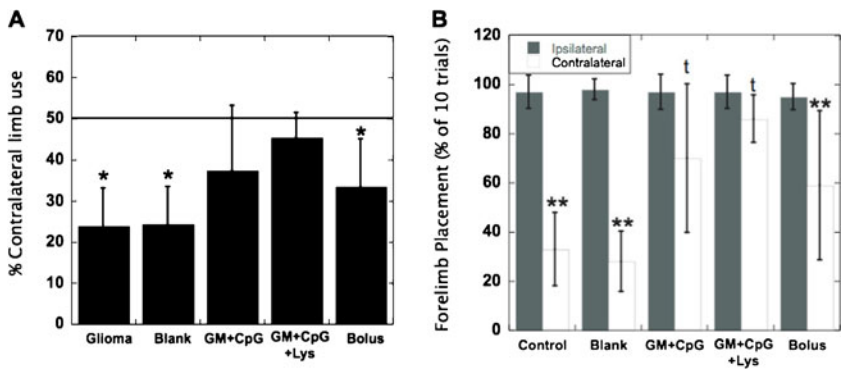

Fig. 4 Therapeutic vaccination with PLG matrices attenuates the neurological deficits associated with uncontrolled growth of C6 glioma cells. As described in the text, 10 days after vaccination, rats were tested for motor function using the cylinder test (left panel) and placement test (right panel). Uncontrolled growth of the C6 glioma (control and blank matrix) produced significant impairments in the ability of the rats to use the contralateral forepaw on both tests. In contrast, those rats receiving vaccines performed normally with use of the ipsilateral and contralateral forelimbs being statistically indistinguishable. Data are presented as mean +/−SD percent contralateral forelimb contacts (left panel) and mean (±SD) number of correct places with the forelimbs ipsilateral and contralateral (right panel) to the tumor-bearing striatum. * = $p < 0.05$ *vs* 50% cutoff for normal performance, ** = $p < 0.05$ *vs* ipsilateral forelimb, and t = $p > 0.05$ *vs* ipsilateral forelimb.

nial glioma. In agreement with previous findings utilizing melanoma models (14,15), the controlled presentation of bioactive factors in the PLG matrix was required for therapeutic benefit, as the bolus injection of GM-CSF, CpG-ODN and glioma lysate did not protect mice from the loss of limb function and did not significantly enhance their survival after glioma-cell injection.

It is worth noting that PLG is FDA approved for use in other medical applications, and GM-CSF and CpG-ODN have an extensive and safe history of clinical testing, which will likely facilitate the clinical development of this system for immunotherapies. The observations of this study suggest that the intracranial implantation of the PLG vaccine safely induced immune-mediated destruction of the tumor, but long-term safety studies should be conducted to ensure the absence of toxicity to normal brain tissue. It is not clear whether the dose of GM-CSF and CpG-ODN is optimal. In this case, however, the matrix system was fabricated with relatively low doses of immune adjuvants, GM-CSF and CpG-ODN, which are not efficacious when delivered in bolus, in a one-time intracranial application to promote significant regression of glioma tumors, and, similarly, this vaccine system may be utilized to deliver other immunostimulants (for example, various TLR agonists), which may enhance their *in vivo* targeting and bioactivity.

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